

DNA ENCODING FLAVIN-ADENINE-DINUCLEOTIDE-DEPENDENT-D-ERYTHRONATE-4-PHOSPHATE-
DEHYDROGENASE, PDXR, AND MICROBIAL PRODUCTION OF VITAMIN B₆

The present invention relates to a DNA encoding a novel flavin adenine dinucleotide (FAD)-dependent D-erythronate 4-phosphate (EN4P) dehydrogenase originated from *Sinorhizobium meliloti*, which is involved in vitamin B₆ biosynthesis, and a recombinant
5 microorganism transformed with a vector having the DNA. It also relates to a process for production of vitamin B₆ by using the recombinant microorganism.

"Vitamin B₆" as used in this invention includes pyridoxol (PN), pyridoxal and pyridoxamine. Vitamin B₆ is a vitamin indispensable to human beings or other animals, and is used as a raw material of medicines or as feed additives.

- 10 Fermentative production of vitamin B₆ by *Sinorhizobium* [also known as *Rhizobium*: De Lajudie et al., Int. J. Syst. Bacteriol. 44:715-733 (1994)] *meliloti* is reported (EP 765,938). But the production efficiency of vitamin B₆ in the process for preparing vitamin B₆ using a microorganism is not so high and it has been required to construct a new microorganism having higher productivity of vitamin B₆.
- 15 4-PHT pathway in *E. coli* is as follows: 4-PHT synthesis starts from D-erythrose 4-phosphate (E4P), which is oxidized by an E4P dehydrogenase to EN4P, further oxidized by an EN4P dehydrogenase to 2-keto-EN4P, and transaminated by an aminotransferase to yield 4-PHT. The three enzymes, E4P dehydrogenase, EN4P dehydrogenase, and aminotransferase, are encoded by three genes, named *epd*, *pdxB*, and *serC*, respectively, and there is
20 only one report on the EN4P dehydrogenase of *E. coli*, which requires NAD⁺ as a coenzyme for the enzyme reaction [Zhao et al., J. Bacteriol. 177:2804-2812 (1995)].

On the other hand, the 4-PHT in *S. meliloti* is synthesized from glycine and glycolaldehyde. But a gene responsible to the reaction has not been identified yet. For the

purpose of identifying the gene, we obtained a vitamin B₆-deficient mutant of *S. meliloti* IFO 14782 that is defective in 4-PHT formation. And we obtained a gene, namely *pdxR*, which complements the vitamin B₆-deficient mutant, and found that *pdxR* encodes a novel FAD-dependent EN4P dehydrogenase.

- 5 According to the present invention, it is possible to improve the production efficiency of vitamin B₆ greatly by fermentation using a microorganism of the genus *Sinorhizobium* having a recombinant plasmid comprising a vector containing *pdxR*. Vitamin B₆ can advantageously be produced in the culture broth by cultivating said microorganisms and can be recovered therefrom in a desired purity.
- 10 The present invention provides a DNA encoding an FAD-dependent EN4P dehydrogenase originating from *Sinorhizobium*; expression vectors carrying a DNA containing the DNA encoding the EN4P dehydrogenase; a transformed host cell carrying the expression vectors; a polypeptide possessing FAD-dependent EN4P dehydrogenase activity produced in the host cell; and a process for producing vitamin B₆ by using the host cell.
- 15 All scientific and technical terms used in this invention have meanings commonly used in the art unless otherwise specified.

The present invention is based on the discovery of a gene designated *pdxR*, which encodes an FAD-dependent EN4P dehydrogenase, which is involved in vitamin B₆ biosynthesis of *S. meliloti* and the use of the gene for improving vitamin B₆ production.

- 20 In the verification process of the invention, we found a gene, named *pdxR*, which complements a vitamin B₆-deficient mutant of *S. meliloti* IFO 14782 (DSM 10226) that requires 4-hydroxy-L-threonine (referred to as 4-HT hereinafter) or vitamin B₆ for the growth. The *pdxR* does not have homology to any genes disclosed as being involved in vitamin B₆ biosynthesis of *E. coli*.
- 25 According to search of the genome database of *S. meliloti* strain 1021 [Galibert et al., Science 293:668–672 (2001)], deduced amino acid sequence of *pdxR* suggested that *PdxR* is one of oxidoreductases such as dehydrogenases, reductases and oxidases. But it is very difficult to predict its substrate for *PdxR*. The *pdxR* gene was found to complement not only a vitamin B₆-deficient mutant of *S. meliloti* IFO 14782 but also a *pdxB*[−] mutant of *E.*
- 30 *coli* lacking an EN4P dehydrogenase. These findings indicate that *PdxR* is involved in 4-PHT pathway of vitamin B₆ biosynthesis in *S. meliloti* and *pdxR* encodes oxidoreductases catalyzing oxidation of EN4P to 2-keto-EN4P.

By further experiments, a gene product encoded by *pdxR* gene of *S. meliloti* in the present invention was found to contain FAD and to catalyze the oxidation of EN4P using an electron acceptor such as cytochrome c, 2,6-dichlorophenolindophenol (DCIP), or ferricyanide, but not such as O₂, NAD⁺ or NADP⁺. Evidence is presented that the *pdxR* gene product is a dehydrogenase with electron acceptors rather than an oxidase and completely different from EN4P dehydrogenase with NAD⁺ of *E. coli*.

The present invention is directed to DNA sequence comprising a DNA sequence which encodes PdxR, which is an FAD-dependent EN4P dehydrogenase involved in vitamin B₆ synthesis, as disclosed in the sequence listing, as well as their complementary strands, or those which include these sequences, DNA sequences which hybridize, preferably under standard conditions, with such sequences or fragments thereof and DNA sequences, which because of the degeneration of the genetic code, do not hybridize under standard conditions with such sequences but which code for polypeptides having exactly the same amino acid sequence.

"Standard conditions" for hybridization mean in this context the conditions which are generally used by a person skilled in the art to detect specific hybridization signals, or preferably so called stringent hybridization and non-stringent washing conditions or more preferably so called moderately stringent conditions or even more preferably so called stringent hybridization and stringent washing conditions. For example, a condition is hybridizing in 2 × SSC and 0.5% SDS at 45C° for 1 hour and washing in 0.1 × SSC and 0.5% SDS at 60C° for 1 hour.

Present invention also includes DNA sequences that are at least 80% to 85%, preferably at least 86% to 90%, more preferably at least 91% to 95%, and particularly preferably more than 95% identical to the DNA sequence according to SEQ ID NO:1 or a fragment therefrom.

The present invention is also directed to polypeptides having FAD-dependent EN4P dehydrogenase activity, which are functional derivatives of the polypeptides of the present case. Such functional derivatives are defined on the basis of the amino acid sequences of the present invention by addition, insertion, deletion and/or substitution of one or more amino acid residues of such sequences, wherein such derivatives still have the activity of the FAD-dependent EN4P dehydrogenase. Such functional derivatives can be made either by chemical peptide synthesis known in the art or by recombinant means on the basis of the DNA sequence as disclosed herein by methods known in the state of the art. Amino acid exchanges in proteins and peptides which do not generally alter the activity of such

molecules are known in the state of the art. The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse.

Furthermore, polypeptides according to the invention include a polypeptide according to
5 SEQ ID NO: 2, in particular those with the activity of FAD-dependent EN4P dehydrogenase, and also those which are at least 80% to 85%, preferably at least 86% to 90%, more preferably at least 91% to 95%, and particularly preferably more than 95% identical to the polypeptide according to SEQ ID NO: 2 and have the activity mentioned.

To express the pdxR or the DNA sequence encoding FAD-dependent EN4P dehydrogenase
10 efficiently, various promoters can be used; e.g., the original promoter existing upstream of the pdxR gene, promoter of antibiotic gene such as β -galactosidase of *E. coli* (lac), promoter of small ribosomal subunit of *S. meliloti* (pS1), trp-, tac-, trc-promoter, and any promoters which can be functional in the hosts consisting of microorganism including bacteria such as *E. coli* and *S. meliloti*.

15 For the object above, other regulatory elements such as a Shine-Dalgarno (SD) sequence (e.g., AGGAGG and so on, including natural and synthetic sequence operable in the host cell) and a transcriptional terminator (inverted repeat structure including any natural and synthetic sequences operable in the host cell) which are operable in the host cell into which the coding sequence will be introduced and used with the above described promoter.

20 A wide variety of host/cloning vector combinations may be employed in cloning the double-stranded DNA. Cloning vector is generally a plasmid or phage which contains a replication origin, regulatory elements, a cloning site including a multiple cloning site and selection markers such as antibiotic resistance genes including resistance genes for ampicillin, tetracycline, chloramphenicol, kanamycin, neomycin, streptomycin, gentamicin,
25 spectinomycin etc (referred to as Ap, Tc, Cm, Km, Nm, Sm, Gm, Sp hereinafter, respectively).

Preferred vectors for the expression of the object gene in *E. coli* are selected from any vectors usually used in *E. coli*, such as pBR322 or its derivatives including pKK223-3, pUC18 and pBluescriptII, pACYC184 or its derivatives, and a vector derived from a broad host
30 range plasmid such as RK2 and RSF1010. A preferred vector for the expression of the object gene in *S. meliloti* is selected from any vectors that can replicate in this microorganism, as well as in a preferred cloning organism such as *E. coli*. The preferred vector is a broad host range vector such as pVK100, pRK290, pLAFR1, and RSF1010 or a vector

containing a replication origin functional in *S. meliloti* and another origin functional in *E. coli*.

To construct a host cell carrying an expression vector, various DNA transfer methods including transformation, transduction, and conjugal mating can be used. The method for constructing a transformed host cell may be selected from the methods well known in the field of molecular biology. Usual transformation and transduction systems can be used for *E. coli*. Conjugal mating system can be widely used in Gram-positive and Gram-negative bacteria including *E. coli* and *S. meliloti*.

Vitamin B₆-deficient mutant that has a mutated point on the pathway leading to 4-PHT can be constructed by transposon mutagenesis.

Transposon is a distinct DNA segment, which has a capacity to move into the genome. One of these transposons, Tn5, is a 5,818-bp composite transposon, consisting of two inverted repeats and three genes conferring resistance to the antibiotics. Tn5 has a low insertional specificity and Tn5 mutagenesis can be performed [Meade et al., J. Bacteriol. 149:114–122 (1982)]. A suicide vector, pSUP2021, which carries Tn5 derived from *Pseudomonas* and is not replicable in *S. meliloti*, can be used to introduce Tn5 insertion into the genome of *S. meliloti* [Simon et al., BIO/TECHNOLOGY 1:784 (1983)]. A helper plasmid, e.g., pRK2013 (ATCC 37159), which helps immobile plasmid transfer into other microorganism, can be used.

Tn5 on the suicide vector can be introduced into *S. meliloti* IFO 14782 by tri-parental conjugal mating in the following manner. *S. meliloti* as a recipient strain, *E. coli* harboring helper plasmid as a helper strain, and *E. coli* harboring donor plasmid as a donor strain are cultivated separately and mixed together. After mixed cultivation on plate, *S. meliloti* that has Tn5 integrated in its genome can be selected on agar plate containing appropriate antibiotics. Among these antibiotic-resistant mutants, mutants that produce vitamin B₆ less or none can be selected by the amount of vitamin B₆, which they produce, with vitamin B₆ detection plate. The less or none vitamin B₆ producing mutants can also be examined about requirement of intermediates on the pathway to vitamin B₆. With these supplement experiments, a mutated point on the pathway can be suggested.

A DNA segment, which complements vitamin B₆ deficient mutant, can be screened from genomic library of *S. meliloti*. Genomic library is comprised of large variety of partially digested chromosomal DNA ligated into cosmid and transferred to vitamin B₆ deficient mutant. Transconjugant complemented with target DNA segment can be obtained by

growth on vitamin B₆-free plate. A DNA sequence of the segment can be determined by methods known in the state of the art.

PdxR is over-expressed to investigate a function of its product in host cell. When the PdxR is prepared to be over-expressed, the polymerase chain-reaction (PCR) method is suitable for adding restriction enzyme recognition site to place pdxR under control of a strong promoter. The primers for PCR are synthesized in accordance with the DNA sequence of pdxR and flanking region, and one primer contains restriction enzyme recognition sequence at its 5' end. The DNA fragment containing pdxR can be amplified by PCR using the primers and chromosomal DNA of *S. meliloti*. Amplified DNA can be ligated into a vector replicable in *E. coli* as described above in a desired structure. A plasmid, wherein amplified DNA is inserted, can be selected and the sequence of amplified region can be ascertained by methods known in the state of the art.

For preparation of the cell-free extract from the cells obtained by cultivation, general method such as sonication, cell breakage in the presence of glass beads or by French press homogenizer can be applied. If desired, treatment with a lytic enzyme such as lysozyme or zymolase at 15°C to 45°C, preferably 20°C to 40°C for 1 to 3 hours can also be applied before the disruption in the above-mentioned way. For example, after centrifugation of culture broth, the resulting cells are washed with 0.85% (w/v) saline and suspended in a buffer such as Tris-HCl buffer, pH 7.5. After cell breakage, the resulting solution is centrifuged to separate the cell debris, and its supernatant can be used as cell-free extract.

The protein concentration was determined by the Lowry method.

EN4P dehydrogenase activity with an electron acceptor can be verified by observing or by measuring spectrophotometrically color change due to formation of reduced form from oxidized form of an electron acceptor such as cytochrome c, DCIP, or ferricyanide when EN4P dehydrogenase in this invention exist together with one of these electron acceptors in appropriate buffer.

The transformed host cell obtained in the present invention is incubated in a medium containing an assimilable carbon source, a digestible nitrogen source, an inorganic salt, and other nutrients necessary for their growth. As a carbon source, e.g., glucose, fructose, lactose, maltose, galactose, sucrose, starch, dextrin, or glycerol may be employed. As a nitrogen source, e.g., peptone, corn steep liquor, soybean powder, yeast extract, meat extract, ammonium chloride, ammonium sulfate, ammonium nitrate, urea, or their mixture thereof may be employed. Further, for trace elements, sulfates, hydrochlorides, or phos-

phates of calcium, magnesium, zinc, manganese, cobalt, and iron may be employed. If necessary, conventional nutrient factors, a trapping agent of phosphate ion, or an antifoaming agent, such as magnesium carbonate, aluminum oxide, allophane, animal oil, vegetable oil, or mineral oil can also be added supplementary in a fermentation medium.

- 5 The pH of the culture medium may be about 5.0 to about 9.0, preferably about 6.5 to about 7.5. The cultivation temperature may be about 10°C to about 40°C, preferably about 25°C to about 35°C. The cultivation time may be about 1 day to about 15 days, preferably about 2 days to about 9 days. In the cultivation, aeration and agitation usually give favorable results.
- 10 After the cultivation, produced vitamin B₆ may also be separated from the culture broth and purified. For this purpose, a process generally used for extracting a certain product from the culture broth may be applied by utilizing various properties of vitamin B₆. Thus, e.g., the cells are removed from the culture broth, the desired substance in the supernatant is absorbed on active carbon, then eluted and purified further with an ion exchange resin.
- 15 Alternatively, the culture filtrate is applied directly to an ion exchange resin and, after the elution, the desired product is recrystallized from mixture of alcohol and water.

A vitamin B₆ deficient mutant, *S. meliloti* 16C18, was obtained by screening of Tn5 insertion mutagenesis from *S. meliloti* IFO 14782 (DSM 10226). The mutant having a mutation point on 4-PHT pathway is necessary for isolation of pdxR gene encoding EN4P dehydro-

20 genase and was deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) in Göttingen (Germany) on September 17, 2002 under accession no. DSM15210 under the Budapest Treaty.

The invention is explained in more detail below with the aid of a few implementation examples. The following text is intended to indicate the materials and methods employed

25 and to support the invention with experimental examples and comparative examples, but it is not construed as being limited thereto.

Example 1

[A] Isolation of vitamin B₆-deficient Tn5 insertion mutant, *S. meliloti* 16C18

Vitamin B₆-deficient mutants were screened from Tn5 insertion mutants of *S. meliloti* IFO 14782 that is a natural high producer of vitamin B₆. Tn5 was introduced into *S. meliloti*

30 IFO 14782 by tri-parental conjugal mating as described below. *S. meliloti* IFO 14782 as a recipient strain was inoculated in 5 ml of liquid LBMC medium consisting of 1 % Tryptone Peptone (Becton Dickinson Microbiology systems, MD, USA), 0.5 % Bacto Yeast

Extract (Becton Dickinson Microbiology systems, MD, USA), 0.5 % NaCl, 0.061 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.036 % $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and incubated with shaking at 30°C at 140 rpm for 16 hours. The culture (400 μl) was transferred into fresh medium and incubated for 6 hours again. *E. coli* HB101 harboring pRK2013 (ATCC 37159) as a helper strain was in-
5 inoculated in 5 ml of liquid LB medium consisting of 1 % Tryptone Peptone, 0.5 % Bacto Yeast Extract, and 0.5 % NaCl containing 50 $\mu\text{g}/\text{ml}$ of Km and incubated with shaking at 37°C at 140 rpm for 16 hours. The culture (100 μl) was transferred into fresh medium and incubated for 6 hours again. *E. coli* HB101 harboring pSUP2021 as a Tn5 donor strain was inoculated in 5 ml of liquid LB medium containing 50 $\mu\text{g}/\text{ml}$ of Km and incubated with
10 shaking at 37°C at 140 rpm for 16 hours. The culture (100 μl) was transferred into fresh medium and incubated for 6 hours again. Each strain was harvested and cells were mixed at 1:1:4 (v/v/v) ratio. The mixture was put on a nitrocellulose filter placed on LBMC agar plate. After this plate was incubated for 20 hours at 30°C, cells on the filter were scratched and suspended in sterilized 0.85% saline. The suspension was diluted appropriately and
15 spread on LBMC plates containing 20 $\mu\text{g}/\text{ml}$ of Nal (to select for *S. meliloti* IFO 14782) and 50 $\mu\text{g}/\text{ml}$ of Nm (to select for Tn5). After incubation of the plates at 30°C for 4 days, about 10,000 colonies appeared on the plates. The frequency of Nm resistant colony was $4.2 \times 10^{-8}/\text{recipient}$.

These colonies were streaked on LBMC plates containing same antibiotics and incubated
20 for 2 days at 30°C. The cells grown on the plates were inoculated on vitamin B₆ assay plates (Table 1) containing *Saccharomyces carlsbergensis* ATCC 9080 as a vitamin B₆ indicator strain. After incubation for 2 days at 30°C, one colony that had no halo of *Saccharomyces carlsbergensis* ATCC 9080 was obtained. The strain was named as *S. meliloti* 16C18. Strain 16C18 did not grow on EMM plate (Table 1), wherein parent strain, *S. meliloti* IFO
25 14782, grew. Further, *S. meliloti* 16C18 grew on EMM plate which was supplemented with 4 mg/L of 4-HT or 400 $\mu\text{g}/\text{L}$ of PN. This result suggests that *S. meliloti* 16C18 is a vitamin B₆ deficient mutant possessing a mutation point on 4-PHT pathway.

Table 1: Media composition (vitamin B₆ assay plate and EMM plate)

Ingredient	vitamin B ₆ assay plate	EMM plate
glucose	50 g	10 g
vitamin free casamino acid	-	8 g
L-arginine	160 mg	-
L-aspartic acid	20 mg	-
L-glutamic acid	200 mg	-
glycine	40 mg	-
L-histidine	80 mg	-
L-isoleucine	180 mg	-
L-leucine	400 mg	-
L-lysine	280 mg	-
L-methionine	80 mg	-
L-phenylalanine	160 mg	-
L-threonine	160 mg	-
L-tyrosine	80 mg	-
L-valine	280 mg	-
KH ₂ PO ₄	550 mg	-
MnSO ₄ ·5H ₂ O	2.5 mg	2.5 mg
MgSO ₄ ·7H ₂ O	125 mg	125 mg
CaCl ₂ ·2H ₂ O	125 mg	125 mg
KCl	425g	425 mg
FeCl ₃ ·6H ₂ O	2.5 mg	250 µg
thiamin hydrochloride	250 µg	250 µg
biotin	8 µg	8 µg
deionized water	1000 ml	1000 ml
bacto-agar (Difco)	15 g	15 g
pH	6.0	6.8

[B] Isolation of the DNA fragment complementing growth of *S. meliloti* 16C18 on EMM plate

- 5 Genomic library of *S. meliloti* IFO 14782 was constructed as a tool to obtain DNA fragment which made *S. meliloti* 16C18 grow on EMM plate without 4-HT or PN.

Chromosomal DNA of *S. meliloti* IFO 14782 was prepared using QIAGEN Genomic-tips (QIAGEN, GmbH, Germany). The chromosomal DNA was digested with *EcoR* I. The partially digested DNA was subjected to agarose gel electrophoresis on 0.6% gel. A piece of gel containing 15-kb~35-kb DNA fragments was cut out and the DNA fragments were
5 eluted from the gel by electrophoresis. Eluted DNA fragments were precipitated with ethanol and recovered by centrifugation. In parallel, 400 ng of plasmid pVK100 was completely digested with *EcoR* I and dephosphorylated with alkaline phosphatase. The treated pVK100 was ligated with above-mentioned chromosomal DNA fragments (1 μ g) of *S. meliloti* IFO 14782 by ligation kit (Takara Bio Inc., Shiga, Japan). The DNA was subjected
10 to *in vitro* packaging of phage (Amersham Biosciences Corp., NJ, USA). Then, *E. coli* ED8767 [Murray et al., Mol. Gen. Genet. 150:53–61 (1977)], which was harvested during exponential growth, was infected with the phage, which contains various DNA fragments of *S. meliloti* IFO 14782. The infected *E. coli* was spread out on LB medium containing 10 μ g/ml of Tc. After incubation for 17 hours at 37°C, approximately 10,000 colonies
15 appeared on plates. These colonies were scratched all together, incubated in LB liquid medium at 37°C for 1 hour, dispensed into small tubes with lids, and stored at –120°C. This genomic library in *E. coli* ED8767 was introduced into *S. meliloti* 16C18 by tri-parental conjugal mating as described below to screen transconjugants, which grew EMM plate without PN.

20 *S. meliloti* 16C18 as a recipient strain was inoculated in liquid LBMC medium and incubated with shaking at 30°C at 140 rpm for 16 hours. The culture (400 μ l) was transferred into fresh same medium and incubated for 6 hours again. *E. coli* HB101 harboring pRK2013 as a helper strain was inoculated in liquid LB medium containing 50 μ g/ml of Km and incubated with shaking at 37°C at 140 rpm for 16 hours. The culture (100 μ l) was
25 transferred into fresh medium and incubated for 6 hours again. Frozen-stored *E. coli* ED8767 harboring genomic library of *S. meliloti* IFO 14782 as a donor strain was inoculated in liquid LB medium containing 10 μ g/ml of Tc and incubated with shaking at 30°C at 140 rpm for 2 hours. After each strain was harvested, they were mixed at 1:1:1 ratio and the mixed cells were put on nitrocellulose filter placed on LBMC agar plate. After incubation
30 for 20 hours at 30°C, cells on the filter were scratched and suspended into sterile 0.85% saline. The suspension was diluted and spread out on EMM plates containing 20 μ g/ml of Nal (to select for *S. meliloti*) and 10 μ g/ml of Tc (to select for the plasmid). After incubation at 30°C for 8 days, 4 colonies appeared. Plasmids in these colonies were prepared, digested with restriction enzymes, and analyzed on agarose gel. Among these plasmids,
35 one plasmid, which has a 23.0-kb insertion, was designated as pSHT09. A 2.5-kb

DNA fragment generated by cleaving pSHT09 with *EcoR* I was ligated into pVK100 that had been opened at *EcoR* I site and the ligation mixture was transformed to *E. coli* HB101 competent cells (Takara Bio Inc., Shiga, Japan). Plasmids in appeared colonies were prepared, digested with restriction enzymes and analyzed on agarose gel. A plasmid, which
5 contains a 2.5-kb insertion, was named pVK-HTS1. This plasmid was transferred into *S. meliloti* 16C18 by tri-parental conjugal mating as described above. *S. meliloti* 16C18 harboring pVK-HTS1 (*S. meliloti* 16C18/pVK-HTS1) grew on EMM plate without PN or 4-HT. This indicates that there is a gene complementing the growth of *S. meliloti* 16C18 within the 2.5-kb DNA fragment.

10 [C] Specification of the complementing gene

DNA sequence of the 2.5-kb fragment, which was obtained in Example 1 [B], was determined with an ALFred DNA sequencer (Amersham Biosciences Corp., NJ, USA). Within the sequence, there was an open reading frame (referred to as ORF hereafter) whose length was 1491-bp (SEQ ID NO: 1). Sequence of the ORF was identical with the CDS region of
15 SMc00985 (951316–952806, complement) in accession number NC_003047. Deduced amino acid sequence of the CDS (SEQ ID NO: 2) was predicted as a putative oxidoreductase, though its substrate had not been identified. Then the ORF was named as *pdxR*. A recombinant plasmid, which carries the *pdxR*, was constructed as follows. The DNA fragment containing *pdxR* and flanking region was amplified from 100 ng of chromosomal
20 DNA of *S. meliloti* IFO 14782 with advantage-HF PCR kit (Clontech Laboratories, Inc. CA, USA) using 10-pmol of two primers, SEQ ID NOs: 3 and 4. One primer for amino-terminal was designed to contain an initiation codon just after the *EcoR* I site. Reaction condition was as follows; after holding 15 sec at 94°C, 25 cycles of 15 sec at 94°C, 15 sec at 50°C, 3 min at 68°C. The resultant 2.2-kb PCR product was inserted into pCRII-TOPO vector
25 with TOPO TA cloning kit (Invitrogen Japan K.K., Japan). It was ascertained that sequence of *pdxR* in amplified region was identical with the corresponding CDS region of SMc00985 (951316–952806, complement) in accession number NC_003047. The 1.7-kb fragment of DNA corresponding to the amplified region was cut out with *EcoR* I and the fragment was recovered from agarose gel with QIAEXII (QIAGEN, GmbH, Germany).
30 The 1.7-kb fragment was ligated to pKK223-3 expression vector (Amersham Biosciences Corp., NJ, USA) that had been opened with *EcoR* I and dephosphorylated by alkaline phosphatase. After transformation of *E. coli* JM109 competent cells (Takara Bio. Inc., Shiga, Japan) with the ligation mixture, plasmids in transformants were prepared, digested with restriction enzyme and analyzed on agarose gel. A recombinant plasmid, pKK-*pdxR*,
35 wherein *pdxR* gene was inserted into the *EcoR* I site of pKK223-3 and into the same

direction of tac promoter on the vector, was obtained. Plasmid pKK-pdxR was prepared with *E. coli* JM109/pKK-pdxR with QIAGEN Midi kit (QIAGEN GmbH, Germany). Thus obtained plasmid pKK-pdxR was digested with *Bam*H I. Resulting 2-kb fragment containing tac promoter and pdxR was purified from agarose gel. The 2-kb fragment was
5 ligated to pVK100 that had been opened with *Bgl* II and dephosphorylated with alkaline phosphatase. *E. coli* HB101 competent cells (Takara Bio Inc., Shiga, Japan) were transformed with this ligation mixture and plasmids in transformants were prepared, digested with restriction enzyme and analyzed on agarose gel. A recombinant plasmid pVK-PdxR, wherein tac promoter and pdxR gene were inserted into the *Bgl* II site of pVK100 as the
10 opposite direction of Km resistant gene, was obtained. Thus obtained pVK-pdxR was introduced into *S. meliloti* 16C18 by tri-parental conjugal mating as described in Example 1 [B] except selection plates, which are LBMC containing 20 mg/L of Nal and 10 mg/L of Tc. Transconjugants grown on selection plates were obtained and plasmids in the transconjugants were ascertained to have the same structure of
15 pVK-pdxR. Then, these transconjugants were streaked on EMM plate containing 50 mg/L of Nm and 10 mg/L of Tc. After incubation at 30°C, all tested transconjugants could grow on the plate, on which *S. meliloti* 16C18/pVK100 could not grow. This indicates that the amplified pdxR placed under tac promoter can complement *S. meliloti* 16C18.

Example 2: Enzyme function and characteristics of a pdxR gene product

20 [A] Complementation of *E. coli* PdxB⁻ mutant by pdxR gene

Thus obtained pKK-pdxR in Example 1 [C] was transformed into *E. coli* WG1012, which is PdxB⁻ mutant (Dempsey, W. B., J. Bacteriol., vol.100, p.295, 1969). Resultant transformants and parent strain, *E. coli* WG1012, were streaked on EMM plates with and without PN. After incubation for 16 hours at 37°C, all tested transformants could grow on
25 EMM plates without PN, on which *E. coli* WG1012 could not grow. This indicates that pdxR gene product of *S. meliloti* has the same function as pdxB gene product in *E. coli*, which converts EN4P to 2-keto-EN4P.

[B] Enzyme reaction system for EN4P oxidoreductase

30 (1) Construction of microorganisms for production of pdxR gene product and preparation of cell-free extract

Then *S. meliloti* 16C18 and one clone of the transconjugants obtained by introduction of pVK-pdxR into *S. meliloti* 16C18 as described in Example 1 [C], *S. meliloti* 16C18/pVK-pdxR, were grown in tubes containing 6 ml of a seed medium composed of 1% glucose,

0.5% Polypepton (Nihon Pharmaceutical Co., Osaka, Japan), 0.2% Bacto Yeast Extract, 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, and 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ at 28°C for 17 hours.

The seed cultures (each 4 ml) were transferred into 500-ml flasks containing 200 ml of a fermentation medium composed of 4% glucose, 4% Polypepton S (Nihon Pharmaceutical Co., Osaka, Japan), 0.8% Bacto Yeast Extract, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and one drop of Actocol (Takeda Chemical Industries, Ltd., Osaka, Japan). The flasks were shaken on a rotary shaker (180 rpm) at 28°C. After cultivation for 72 hours, 35.3 and 35.5 g of wet cells were obtained from 2 liters of the culture broth of *S. meliloti* 16C18 and *S. meliloti* 16C18/pVK-pdxR, respectively, by centrifugation at $10,400 \times g$ for 10 minutes, and the cells were washed with 200 ml of 0.85% NaCl solution twice and stored at -30°C until use.

The frozen cells were thawed slowly, suspended with 200 ml of 10 mM Tris-HCl buffer, pH 7.5. The suspension was passed through a French pressure cell at 800 kg/cm^2 . The homogenate was centrifuged to remove the cell debris at $37,000 \times g$ for 90 minutes and then each 165 ml of supernatants was used as cell-free extract (CFE) and the protein contents in *S. meliloti* 16C18 and *S. meliloti* 16C18/pVK-pdxR were 5,030 and 5,042 mg, respectively.

To confirm the expression of PdxR, the each CFE (5 ml) was dialyzed overnight against 4 liters of 10 mM Tris-HCl buffer, pH 7.5, and subjected to SDS-PAGE on 10 % (w/v) gels and stained with Coomassie Brilliant Blue (Rapid Stain CBB Kit, nacalai tesque Japan). Overexpression of polypeptide with an expected molecular size (53.0 kDa) was detected in *S. meliloti* 16C18/pVK-pdxR but not in *S. meliloti* 16C18. Therefore, the CFEs were used for further study.

(2) EN4P oxidoreductase activities of cell-free extract

EN4P was prepared from E4P (Sigma Chemical Co., St. Louis, MO., USA) by the bromine oxidation according to the method of Horecker [Methods in Enzymology 3:172-174 (1957)]. The product was confirmed as m/z 215 $(\text{M-H})^-$ by negative fast atom bombardment-mass spectrometric analysis (a Jeol SX-102/102 mass spectrometer) and chromatographic properties of the product was identical with those of material prepared by the method of Zhao et al. [J. Bacteriol. 177:2804-2812 (1995)].

Enzyme reaction system for PdxR was studied using CFE prepared in Example 2 [B] (1). EN4P oxidase activity with oxygen in CFE was examined by observation of color change (from colorless to pinkish) due to formation of hydrogen peroxide by a modification of 4-aminoantipyrine peroxidase system used for sarcosine oxidase assay by Inouye et al. as

follows. Reaction mixture (150 μ l) contained 50 mM potassium phosphate buffer, pH 7.0, 2.3 mM EN4P, 0.47 mM 4-aminoantipyrine (Wako Pure chemical Industries, Ltd., Osaka, Japan), 2 mM phenol (Wako Pure chemical Industries, Ltd., Osaka, Japan), 19 U horse-radish peroxidase (Sigma Chemical Co.), and CFE (0.16 mg protein), and color changes of
5 reaction mixtures were observed at room temperature (21°C) or at 37°C. But, after 10 min, no color change occurred at the both temperatures in the reaction mixtures with CFE prepared from cells of *S. meliloti* 16C18 or *S. meliloti* 16C18/pVK-pdxR, whereas hydrogen peroxide standard solutions gave pinkish colors with intensity corresponding to the concentrations. From the results, the both CFEs were determined to have no EN4P oxidase
10 activity with oxygen.

EN4P dehydrogenase activity with NAD⁺ or NADP⁺ in CFE was determined by the formation of NADH or NADPH, which absorbs at 340 nm. Reaction mixture (150 μ l) for assaying EN4P dehydrogenase activity contained 33 mM potassium phosphate buffer, pH 7.0, 2.3 mM EN4P, 1 mM NAD⁺ or NADP⁺, and CFE prepared from cells of *S. meliloti* 16C18
15 or *S. meliloti* 16C18/pVK-pdxR (each 0.16 mg protein). The increase of A₃₄₀ was monitored at room temperature (21°C) for 1 min in a UV-VIS recording spectrophotometer UV 2200 (Shimadzu Instruments, Inc., Kyoto, Japan), but the increase of A₃₄₀ was not observed in enzyme reaction mixtures with any CFEs. Therefore, the both CFEs were determined to have no NAD⁺ or NADP⁺ dependent EN4P dehydrogenase activity.

20 EN4P dehydrogenase activity with an electron acceptor in CFE was examined at room temperature (21°C) by color change due to formation of reduced form from oxidized form of an electron acceptor. Reaction mixture (150 μ l) contained 33 mM potassium phosphate buffer, pH 7.0, 2.3 mM EN4P, CFE (0.16 mg protein), and an electron acceptor such as 2,6-dichlorophenolindophenol (0.091 mM) (Wako Pure chemical Industries, Ltd., Osaka,
25 Japan) (referred to as DCIP hereinafter) or ferricyanide (1.3 mM) (Wako Pure chemical Industries, Ltd., Osaka, Japan). Discoloration of DCIP and ferricyanide occurred within 30 seconds and 1 minute respectively only when CFE prepared from cells of *S. meliloti* 16C18/pVK-pdxR was used in enzyme reaction system containing EN4P, although the discoloration of both electron acceptors never occurred in enzyme reaction system without
30 EN4P in the presence of CFE of *S. meliloti* 16C18/pVK-pdxR or in enzyme reaction system with and without EN4P in the presence of CFE of *S. meliloti* 16C18 (Table 2). These results indicate that a pdxR gene product oxidizes EN4P with electron acceptor such as DCIP or ferricyanide.

Table 2: Color change of electron acceptors in reaction mixture

	<i>S. meliloti</i> 16C18	<i>S. meliloti</i> 16C18/pVK-pdxR
DCIP ¹⁾ + EN4P ²⁾	No change	Discoloration within 30 sec.
DCIP - EN4P	No change	No change
ferricyanide + EN4P	No change	Discoloration within 1 min.
ferricyanide - EN4P	No change	No change

DCIP¹⁾: 2,6-dichlorophenolindophenol, EN4P²⁾: D-erythronate 4-phosphate.

Furthermore, possibility of cytochrome c instead of DCIP or ferricyanide in above-mentioned enzyme reaction system of EN4P dehydrogenase with an electron acceptor was examined by measuring change of absorption spectra in 400–600 nm spectral regions due to formation of reduced form from oxidized form of cytochrome c at room temperature (21°C). Reaction mixture (150 μ l) contained 33 mM potassium phosphate buffer, pH 7.0, 2.3 mM EN4P, 80 μ M oxidized form of cytochrome c (from equine heart, Sigma Chemical Co.) as an electron acceptor, and CFE (0.16 mg protein). Absorption spectra of the reaction mixture of zero time and after 2, 5, 10, 15 and 20 min at room temperature (21°C) were monitored with a UV-VIS recording spectrophotometer UV 2200. The absorption spectrum of zero time had only a broad peak at 525 nm, but spectra after 2 min appeared a new peak at 550 nm corresponding to reduced form of cytochrome c, and the absorption in the 550 nm region after 5, 10, 15 and 20 min was increased with time course. This result indicates that cytochrome c also works as electron acceptor in enzyme reaction system of EN4P dehydrogenase.

[C] Properties of EN4P dehydrogenase

(1) Purification of EN4P dehydrogenase with electron acceptors

The pdxR gene product having EN4P dehydrogenase activity with electron acceptors in CFE was purified by the following chromatography.

(Q Sepharose HP chromatography)

The CFE (3,055 mg proteins) of *S. meliloti* 16C18/pVK-pdxR obtained in Example 2 [B] (1) was applied to a Q sepharose HP column (44 mm in diameter and 12.5 cm in height; Amersham Pharmacia Biotech, Uppsala, Sweden), which was equilibrated with 10 mM Tris-HCl buffer, pH 7.5. After washing with 100 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 0.1 M KCl, it was chromatographed with a linear gradient of KCl 0.1 to 0.4 M (total volume, 800 ml). After a small portion of each fractionated effluent was dialyzed overnight against of 10 mM Tris-HCl buffer, pH 7.5, enzyme activity was monitored by

measuring the decrease in absorbance at 600 nm at room temperature (21°C) due to the formation of reduced DCIP. Assay mixtures (150 μ l) contained 33 mM potassium phosphate buffer, pH 7.0, 2.3 mM EN4P, 0.091 mM DCIP, and 2 μ l of dialyzed samples. The active fraction was eluted at the concentration of 0.37 M KCl, collected and dialyzed overnight against 10 mM Tris-HCl buffer, pH 7.5.

(Resource Q chromatography)

The dialyzed active fraction (58 mg proteins) obtained in Q sepharose HP chromatography was chromatographed with a Resource Q column 6 ml column (Amersham Pharmacia Biotech, Uppsala, Sweden), which was equilibrated with 10 mM Tris-HCl buffer, pH 7.5. After washing with 70 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 0.1 M KCl, it was chromatographed with a linear gradient of KCl 0.1 to 0.4 M (total volume, 120 ml). After dialysis of each effluent against 10 mM Tris-HCl buffer, pH 7.5, enzyme activity was measured by the same method described in Q sepharose HP chromatography. The active fraction was eluted at the concentration of 0.22 M KCl, collected and dialyzed overnight against 10 mM Tris-HCl buffer, pH 7.5.

(HiPrep 16/60 sephacryl S-200HR chromatography)

The dialyzed sample from the previous step was concentrated by ultrafiltration (Centriplus YM-10 and followed by Microcon YM-10 concentrators, Amicon Inc., Beverly, Mass., USA) to 300 μ l. The sample (21.6 mg proteins) was applied to a HiPrep 16/60 sephacryl S-200HR column (16 mm in diameter and 60 cm in height; Amersham Pharmacia Biotech, Uppsala, Sweden), which was equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM KCl. After dialysis of each effluent against 10 mM Tris-HCl buffer, pH 7.5, enzyme activity was measured by the same method described in Q sepharose HP chromatography. The active fraction was subjected to SDS-PAGE on 10% (w/v) gels and stained with Coomassie Brilliant Blue. The analysis indicated that the active fraction was almost homogeneous and had a polypeptide with a molecular weight of 53 kDa, which coincided with the molecular weight of the gene product deduced from a DNA sequence of pdxR.

(2) Characterization of flavin binding to PdxR protein

To characterize a flavin binding to PdxR protein, ammonium sulfate fractionation was applied in the finally purified enzyme solution prepared in Example 2 [C] (1) according to a method of Cammack [Biochem. J. 109:45-46 (1968)]. The enzyme solution (1 ml, containing 4.74 mg proteins) was dialyzed overnight against 1 liter of 80% (w/v) ammonium sulfate solution and its resultant precipitate in a dialysis membrane was collected by centrifugation at $34,800 \times g$ for 30 min, dissolved in 0.5 ml of 10 mM Tris-HCl, pH 7.5, and

dialyzed overnight against 4 liters of the same buffer. The volume of dialyzed enzyme solution was brought to 1 ml with 10 mM Tris-HCl, pH 7.5.

Discoloration of DCIP in the enzyme reaction mixture containing 33 mM potassium phosphate buffer, pH 7.0, 2.3 mM EN4P, purified PdxR treated with ammonium sulfate (2.37 μ g proteins), 0.091 mM 2,6-dichlorophenolindophenol, and flavin mononucleotide (FMN) (Sigma Chemical Co.) or FAD (Sigma Chemical Co.) with various concentrations was observed at room temperature (21°C). The degree of discoloration was judged at 3 minutes.

From the test, discoloration of DCIP occurred in reaction mixtures with FAD concentrations greater than 0.014 μ M, but did not occur in those without flavin and with any FMN concentrations as shown in Table 3. These results indicate that FAD is a coenzyme of EN4P dehydrogenase, PdxR, of *S. meliloti*.

Table 3: Effect of flavin on discoloration of DCIP in reaction mixture

Final conc. (μ M)	Discoloration degree	
	FAD	FMN
0	—	—
0.014	+	—
0.059	++	—
0.24	+++	—
0.95	++++	—
0.38	+++++	—
15.2	+++++	—
61	+++++	—

15 (3) Substrate specificity of PdxR

Substrate specificity of PdxR was examined using the finally purified enzyme solution prepared in Example 2 [C] (1). The standard enzyme assay was performed as follows: the basal reaction mixture (total volume 150 μ l) consisting of 33 mM potassium phosphate buffer (pH 7.0), 2.4 μ g proteins, 0.091 mM DCIP, and water up to a total volume of 144 μ l, and was incubated for 1 minute at room temperature (21°C). Then, 6 μ l of 57 mM substrates solution listed in Table 4 was added to give a final concentration of 2.28 mM, and the whole was incubated at room temperature. The decrease in A_{600} was monitored at

21°C for 1 minute for EN4P dehydrogenase read with a UV-VIS recording spectrophotometer UV 2200. One unit of the enzyme activity was defined as the amount of enzyme reduced 1 nmol of DCIP for 1 minute under the action of EN4P dehydrogenase in the assay system described above. From the test, PdxR oxidized only EN4P, and did not
 5 oxidize other α -hydroxy acids tested.

Table 4: Substrate specificity of PdxR

Substrates	Activity (nmol/min/mg protein)
None	0
EN4P	946
glycolate	0
glyoxylate	0
D(+) glyceric acid	0
L-(+)-lactic acid	0
D,L-lactate	0
D,L-isocitrate	0
L-malic acid	0
D-erythronate	0
glycolic acid 2-phosphate	0
D-(-)-glyceric acid 3-phosphate	0

Example 3: Production of vitamin B₆ by recombinant *S. meliloti*

pVK-pdxR obtained in Example 1 [C] was introduced into *S. meliloti* IFO 14782 by tri-
 10 parental conjugal mating as described in Example 1 [C]. Transconjugants grown on LBMC containing 20 mg/l of Nal and 10 mg/L of Tc were obtained. Plasmids in the transconjugants were ascertained to have the same structure of pVK-pdxR. Thus obtained *S. meliloti* IFO 14782/pVK-pdxR and the parent strain, *S. meliloti* IFO 14782, were incubated on a LBMC agar plate at 30°C for 48 hours, and a loopful of each strain was inocu-
 15 lated to tubes containing 8 ml of a seed medium composed of 1% glucose, 1% corn steep liquor (Nihon Syokuhin Kako Co., Ltd., Tokyo, Japan), 0.2% Bacto Yeast Extract, 0.05% MgSO₄·7H₂O, 0.001% MnSO₄·5H₂O, and 0.001% FeSO₄·7H₂O, pH 7.0, and then the tubes were shaken on a reciprocal shaker at 275 rpm at 30°C.

After shaking for 17 hours, each 4 ml of culture broth was transferred to 500-ml flasks with
 20 two baffles containing 200 ml of a production medium composed of 6% glucose, 4% corn steep liquor, 0.8% Bacto Yeast Extract, 0.05% MgSO₄·7H₂O, 0.05% MnSO₄·5H₂O, 1%

Allophosite (Shinagawa Chemicals Co., Ltd., Tokyo, Japan), and 0.025% Actocol, pH 6.8, and shaken on a rotary shaker at 180 rpm at 30°C.

After cultivation for 7 days, contents of vitamin B₆ in the supernatant of each culture broth were quantified by high pressure liquid chromatography (referred to as HPLC hereinafter) and produced vitamin B₆ was calculated by the internal standard method with 4'-deoxy-
 5 pyridoxol as described below. To prepare the samples for HPLC, 200 μ l of 500 mg/l of 4'-deoxypyridoxol as internal substance, 50 μ l of 60% perchloric acid, and 500 μ l of water were mixed with 500 μ l of the standard solutions of pyridoxol (0–100 mg/L) or of the supernatant from the culture broth. Then the mixture was put on ice for 10 min. After
 10 centrifugation at 18,000 \times g for 10 min, a portion of the mixture was loaded on the following column. The analytical conditions were as follows: column, Capcell pak C18 SG120 (4.6 \times 250 mm) (Shiseido Co., Ltd., Tokyo, Japan); mobile phase, 0.1 M sodium perchlorate, 0.1 M potassium phosphate, and 2% acetonitrile (pH 3.5); column temperature, 25–26°C; flow rate, 1.0 ml/min; and detector, ultraviolet (UV) (at 292 nm). As a
 15 result, *S. meliloti* IFO 14782/pVK-pdxR produced 76 mg of pyridoxol per liter and was about 1.2 times higher than the parent, *S. meliloti* IFO 14782 (Table 5).

Table 5: Pyridoxol productivities of *S. meliloti* IFO 14782 and *S. meliloti* IFO 14782/pVK-pdxR

Microorganism	Pyridoxol (mg/L)	Magnitude of increasing
<i>S. meliloti</i> IFO 14782 (DSM 10226)	63	1.0
<i>S. meliloti</i> IFO 14782/pVK-pdxR	76	1.2

20 Example 4: Separation of vitamin B₆ from cultural broth

Produced vitamin B₆ was recovered from the culture broth of *S. meliloti* IFO 14782/pVK-pdxR prepared in the same cultural conditions as described in Example 3. Pyridoxol at each purification step and the concentration was followed by HPLC as described in Example 3.

25 Two liters of the 168-hour culture broth containing 74 mg/L of vitamin B₆ was centrifuged at 7,500 rpm for 10 min. The pH of the resultant supernatant was adjusted to 3.1 with 1N HCl, and then the supernatant was applied to a column (5.5 \times 15 cm) packed with 350 ml of Amberlite CG 120 (H⁺ form, 100–200 mesh, Rohm and Haas Company, Philadelphia, Pennsylvania, USA). The column was washed with 500 ml of deionized
 30 water and then eluted with 5% ammonium hydroxide. The vitamin B₆ fractions were concentrated under reduced pressure. The residue thus obtained was dissolved in 10 ml of

deionized water, and the solution was charged on a column (5.5×16 cm) packed with 380 ml of Dowex 1×4 (OH^- form, 200–400 mesh, Dow Chemical Co., Ltd., Midland, Michigan, USA), and then washed with 500 ml of deionized water. The column was then eluted with 0.1 N HCl. The fractions containing pyridoxol were concentrated to small
5 volume under reduced pressure. After the solid residue was dissolved in small amount of hot ethanol, the solution was kept standing at 4°C overnight. The resultant precipitates were collected by filtration and dried in vacuum to obtain 119 mg of crude crystals. It was recrystallized from ethanol to obtain 91.6 mg of white crystals having a melting point of 160°C . The infrared absorption, UV absorption, and NMR spectrum of the product of the
10 product coincided with those of authentic pyridoxol.